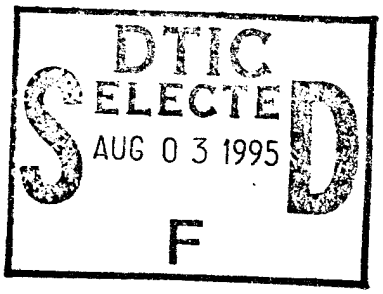
Technical Report 9501

DETERMINATION OF N-NITROSO-N-ETHYLUREA (ENU) IN SALT WATER
BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)



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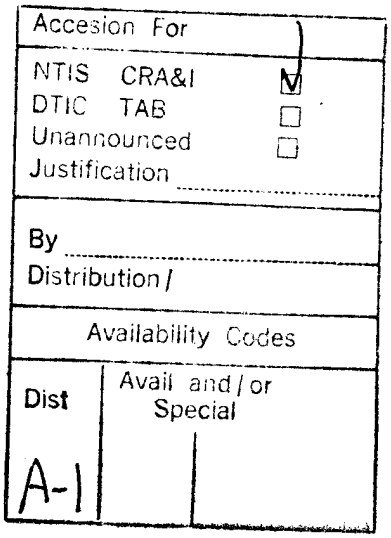

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13. ABSTRACT (Maximum 200 words)

A liquid chromatographic method has been developed for the analysis of N-Nitroso-N-ethylurea (ENU) in salt water by high performance liquid chromatography (HPLC). Samples are injected directly onto a HPLC without sample preparation. Separation of ENU from possible interferences found in salt water was achieved using a C-18 DB column and a mobile phase containing methanol and water. A programmable ultraviolet detector was used to monitor the effluent for ENU. The stability of ENU in the sample matrix is examined at various concentrations and various pH values.

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INTRODUCTION AND OBJECTIVES

The U.S. Army Biomedical Research and Development Laboratory, Research Methods Branch (USABRDL) in cooperation with the National Institute of Environmental Health Sciences (NIEHS) have been involved in tests to investigate the transgenic mummichog (fundulus heteroclitus) as a model for in vivo genotoxicity.[1] N-Nitroso-N-ethylurea (ENU) is a well known alkylating agent and produces central nervous specific tumors in several species.[2] ENU is the test substance being used to determine the feasibility of using this species for this model. Exposures will be performed in salt water, therefore it was necessary to develop a high performance liquid chromatography (HPLC) method for the analysis of ENU in salt water and determine the stability of ENU under the conditions used to expose the mummichog.

ENU is a thermally labile compound that decomposes at 104°C. This property prohibits the use of gas chromatography as an analytical technique, due to the relatively high temperatures required for separation. The ability of HPLC to be used as a method of separation at temperatures below the point where the thermal decomposition of ENU occurs makes HPLC the preferred technique for analysis. Direct injection and a relatively short run time allow for the rapid analysis of salt water samples. The method was developed for analyzing ENU samples in salt water at levels ranging from 0.6 to 100 mg/L.

METHODS AND MATERIALS

ANALYTICAL INSTRUMENTATION

A Hewlett Packard 1050 series HPLC equipped with variable wavelength detector, autosampler, and Hewlett Packard 3396A integrator (Hewlett Packard, Avondale, PA) was used throughout the study. The UV detector was set at 230 nm. The solvent delivery system was programmed to deliver 20 percent methanol/water eluent at a flow rate of 1.5 mL/minute. A Supelco™ DB-18 column (25 cm x 0.46 cm i.d., 5 micron particle size, Supelco, Bellefonte, PA) fitted with a Waters™ C-18 Guard Column (Waters, Milford, MA) was used for the separation of ENU. The column was maintained at 30°C using a BAS™ LC-22A column heater (Bioanalytical Systems, W. Lafayette, IN). The injection volume was 15 µL. An Endocal™ RTE-9DD refrigerated circulating bath (Neslab Instruments, Inc., Portsmouth, NH) was set at 25°C and used for the determination of the stability of ENU in various aqueous media.

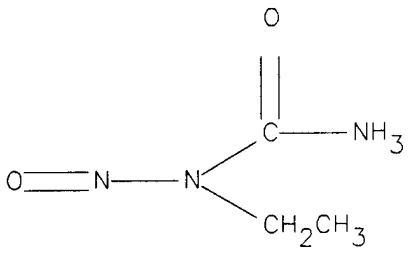
REAGENTS AND MATERIALS

The methanol and acetonitrile used were HPLC grade from Burdick and Jackson (Muskegon, MI). Reagent grade water was obtained with a Barnsted Nanopure™ water purification system (Barnsted/Thermolyne corp., Debuke, IA). N-Nitroso-N-ethylurea containing 18 percent water and 2 percent acetic acid was purchased from Sigma Chemical Co. (Cat no. N 8509, St. Louis, MO). Salt water was prepared by adding 20 g of Instant Ocean™ (Aquarium Systems, Mentor, OH) in 1 L reagent grade water.

The structural formula and other pertinent data for ENU follows:

CAS Registry Number: 759-73-9
RTECHS Reference Number: YT3150000
M.W.: 117.13
Chemical Formula: C3-H7-N3-O2
Description: Yellow-Pink Crystals
Melting Point: 219°F (104°C) Decomposes
Solubility : 1.3 % in water, polar organic solvents
Synonyms: Carbamide, N-Ethyl-N-nitroso-;
1-Ethyl-1-nitrosourea; N-Ethyl-N-nitrosourea; ENU; NEU;
Nitrosoethylurea; NSC 45403; RCRA U176; OHS15780

Structure:



SAMPLE PREPARATION

Due to the unstable nature of ENU, samples were analyzed immediately after they were received in the laboratory. The samples were collected in 2 mL amber glass sample vials and sealed with Teflon™ crimp seals (Hewlett Packard, Avondale, PA). Samples of water containing ENU in concentrations ranging from 100 to 0.2 mg/L were analyzed by direct injection onto a HPLC equipped with a UV spectrophotometer. Water samples containing ENU in excess of 100 mg/L were diluted with reagent grade water to obtain a concentration of 0.6 - 100 mg/L.

PREPARATION OF STOCKS AND STANDARDS

An ENU stock solution was prepared by dissolving 0.050 g of ENU in 100 mL of acetonitrile to give the final concentration of 400 mg/L. Aliquots of the stock solution were diluted in acetonitrile to prepare 0.6, 2, 20, 40, and 100 mg/L working standards. Stocks and standards were prepared fresh daily.

CALCULATIONS

The peak areas of the standards were plotted against their concentrations. From a regression fit, the equation of a straight line was obtained. From the peak area of the ENU sample, its concentration was determined.

$$(\text{mg/L})\ \text{sample} = \frac{a-b}{m}$$

Where, a = peak area
b = y-intercept
m = slope

RESULTS AND DISCUSSION

A chromatogram of a salt water sample containing 1 mg/L ENU is shown in figure 1. The mobile phase and instrumental conditions are listed in the methods section. The detection limit was defined as the lowest concentration that can be reproduced ten times with a relative standard deviation of not more than ten percent. The detection limit was determined by analyzing ten replicates of a standard of ENU prepared in acetonitrile. The mean was 0.33 mg/L with a standard deviation of 9.4% with an upper 95% confidence limit of 0.35 mg/L and a lower 95% confidence limit of 0.31 mg/L. This detection limit may be extended downward, if necessary, by increasing the injection volume. Prior to an analysis, standard solutions were injected onto the HPLC. Peak areas of the standards were plotted against their concentrations to obtain a standard curve as shown in figure 2. Salt water samples containing ENU in concentrations ranging from 0 to 100 mg/L were analyzed by direct injection onto the calibrated HPLC. The concentrations were determined by linear regression analysis.

From preliminary analytical results, ENU was proving to be very unstable in salt water. ENU was dissolved in salt water, deionized water and well water to compare the stability in various matrices. The sample volume for the test was set at 100 mL and the temperature of the water was maintained at 25°C using a sealed jacketed flask attached to a refrigerated recirculating bath. A 1000 mg/L ENU standard prepared in deionized water. The salt water was spiked with this standard to compare the stability at the test concentrations used to expose the mummichog (6, 12.5, 25 ,50 and 100 mg/L).[1] Two replicates of each concentration were analyzed and the results are shown in figures 3 to 7. The stability of the stock of 1000 mg/L ENU was determined over 24 hours by periodically injecting 1.5 µL of a 1000 mg/L ENU stored in an amber glass sample vial at room temperature. The results of these analyses are shown in figure 8. A solution containing 12.5 mg/L ENU in deionized water showed a slow degradation over a 7 day period, as shown in figure 9. Several aquatic species being cultured by USABRDL are fresh water species and are maintained in well water. Figure 10 shows that ENU in well water degrades in a similar manner to salt water.

From these stability studies it was shown that ENU degrades rapidly in salt and well water, but did not degrade rapidly in deionized water. This rapid decomposition shown in these figures can be attributed to the pH of the salt and well water. Salt and well water averaged a pH of 7.8 over a four month period. The acetic acid used as a preservative in ENU gave the 1000 mg/L stock of ENU an acidic pH. It has been stated that spills of ENU

have been treated with weak bases.[3] The decomposition of ENU at various pH values was determined using phosphate buffers prepared at pH values of 6, 7, and 8. The buffers were placed in a jacketed flask at 25°C and spiked to obtain a final concentration of 50 mg/L. Aliquots of the buffers were analyzed at 10 minute intervals and the concentrations were calculated. Time was plotted against the log of each concentration as shown in figure 11. This plot shows that at an acidic pH of 6 the concentration of ENU remained reasonably constant and increasing the pH dramatically increased the rate of decomposition.

In order to insure that the exposures of mummichogs began with the expected initial starting concentration, salt water spikes were prepared at a concentrations near the high and low exposure levels. A 990 mg/L stock of ENU was prepared in reagent grade water. This stock was diluted 1:10 and 1:200 in salt water to prepare high and low spikes. Five replicates of each stock were prepared. Immediately after the preparation of each spike, the sample was injected. The precision and recovery data is shown in table 1.

Table 1. Precision and Recovery of ENU in Salt Water

	Low Spike	High Spike
Spike Concentration	4.95 mg/L	99.0 mg/L
Average	4.09 mg/L	84.1 mg/L
Standard Deviation	0.213	4.62
Relative Standard Deviation	5.21	5.49
Spike Recovery %	82.8	85.8

CONCLUSION

The instrumental conditions used provided a rapid method for the separation of ENU from interferences in salt water. Good sensitivity was provided by monitoring the column effluent at 230 nm. The ability of HPLC to analyze the salt water by direct injection allows the analysis of samples in a relatively short period of time after preparation. This is essential due to the rapid rate of decomposition of samples prepared in salt water. The decomposition that ENU undergoes in basic solutions, including salt water, was significant. Exposures should be performed in at an acidic pH if possible and as rapidly as possible after preparation of any solution used in an exposure to provide expected initial concentration.

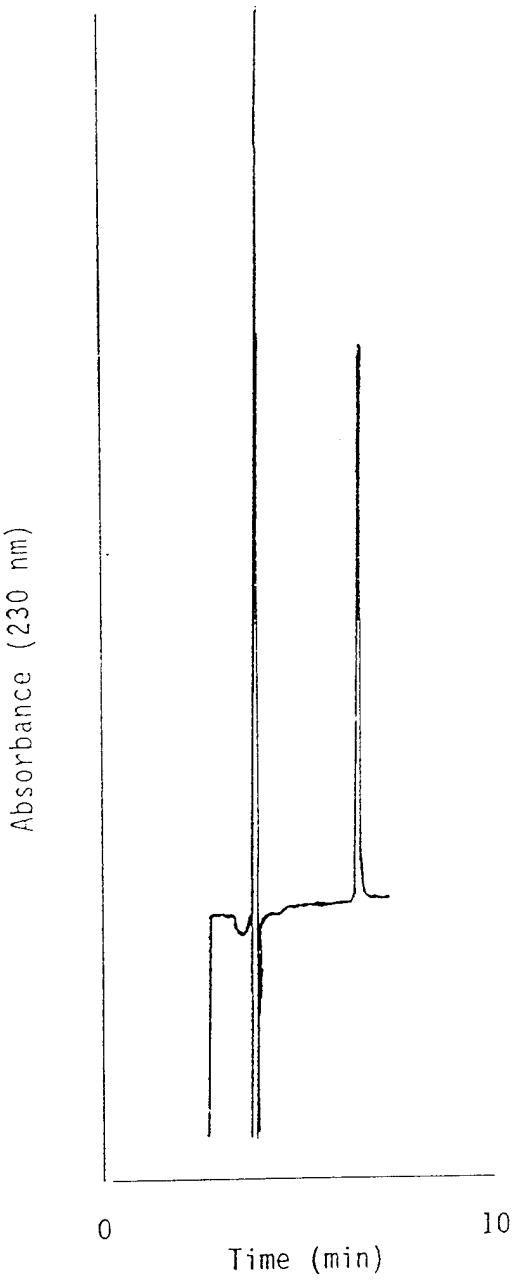


Figure 1. HPLC Chromatogram of a salt water sample containing 1 mg/L N-nitroso-N-ethylurea.

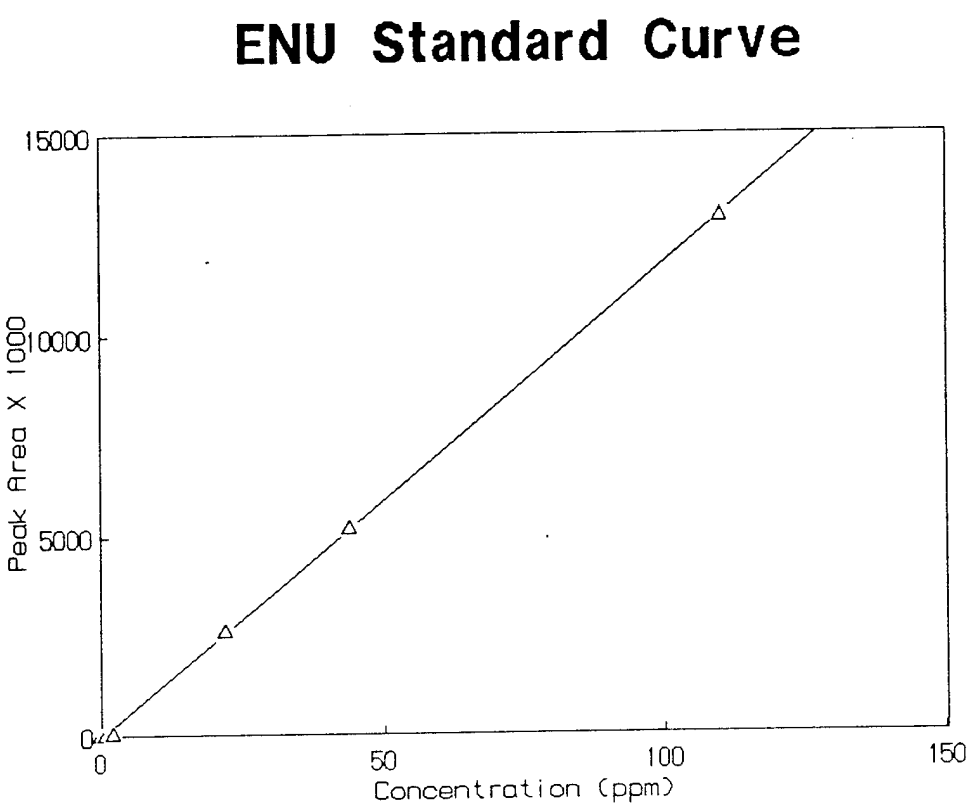


Figure 2. A plot of a N-Nitroso-N-ethylurea standard curve.

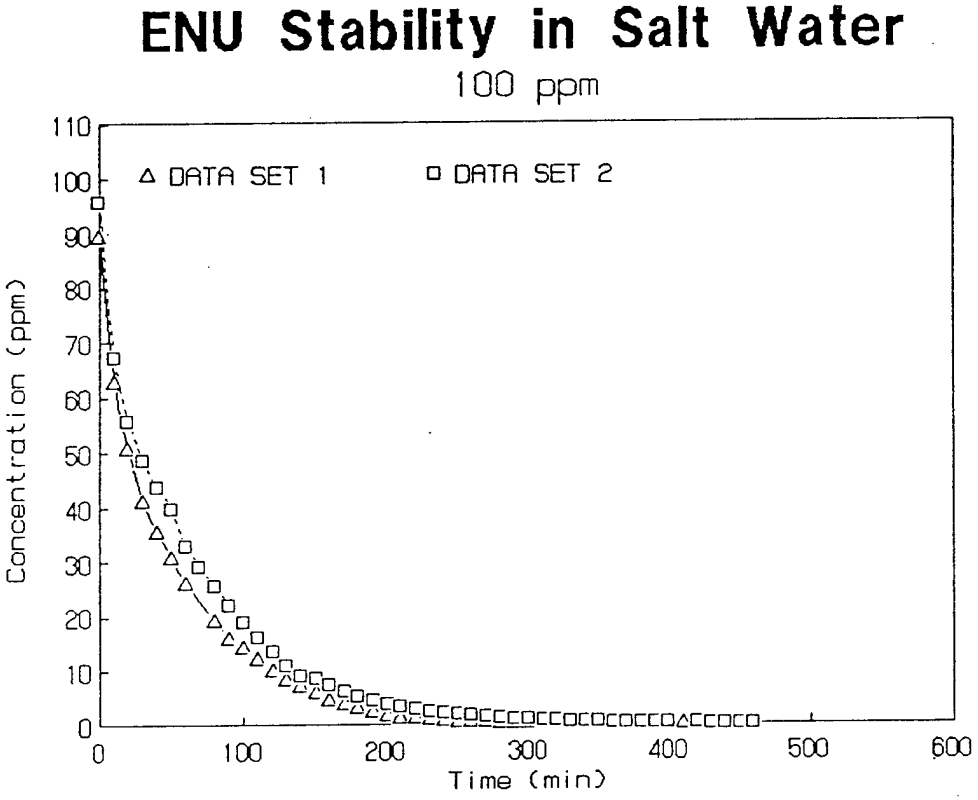


Figure 3. A plot of the stability of a salt water sample spiked with 100 mg/L N-Nitroso-N-ethylurea.

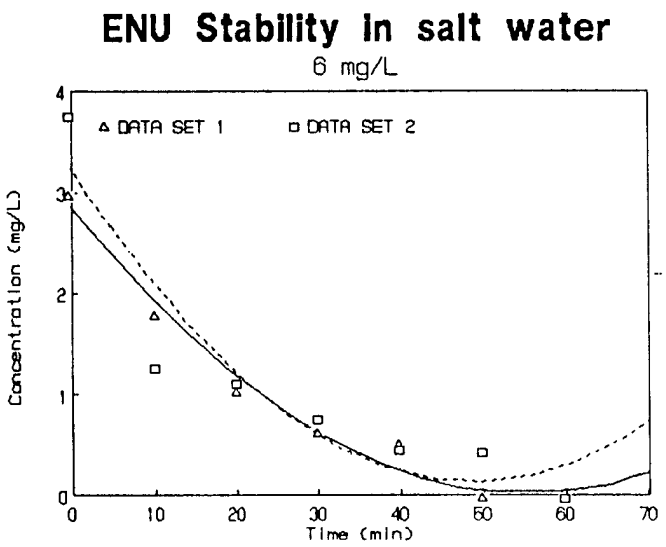


Figure 4. A plot of a salt water sample spiked with 6 mg/L N-Nitroso-N-ethulurea.

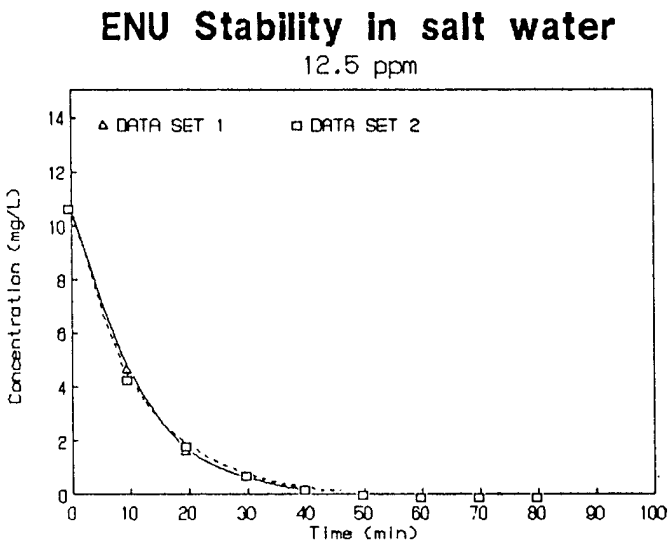


Figure 5. A plot of a salt water sample spiked with 12.5 mg/L N-Nitroso-N-ethulurea.

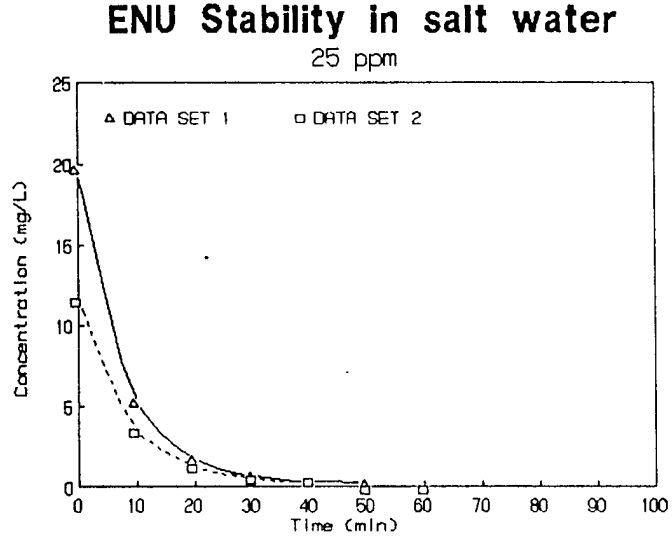


Figure 6. A plot of a salt water sample spiked with 25 mg/L N-Nitroso-N-ethulurea.

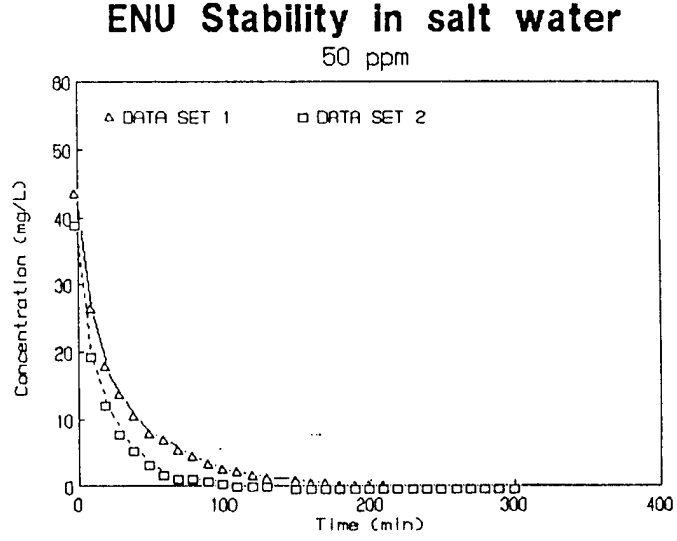


Figure 7. A plot of a salt water sample spiked with 50 mg/L N-Nitroso-N-ethulurea.

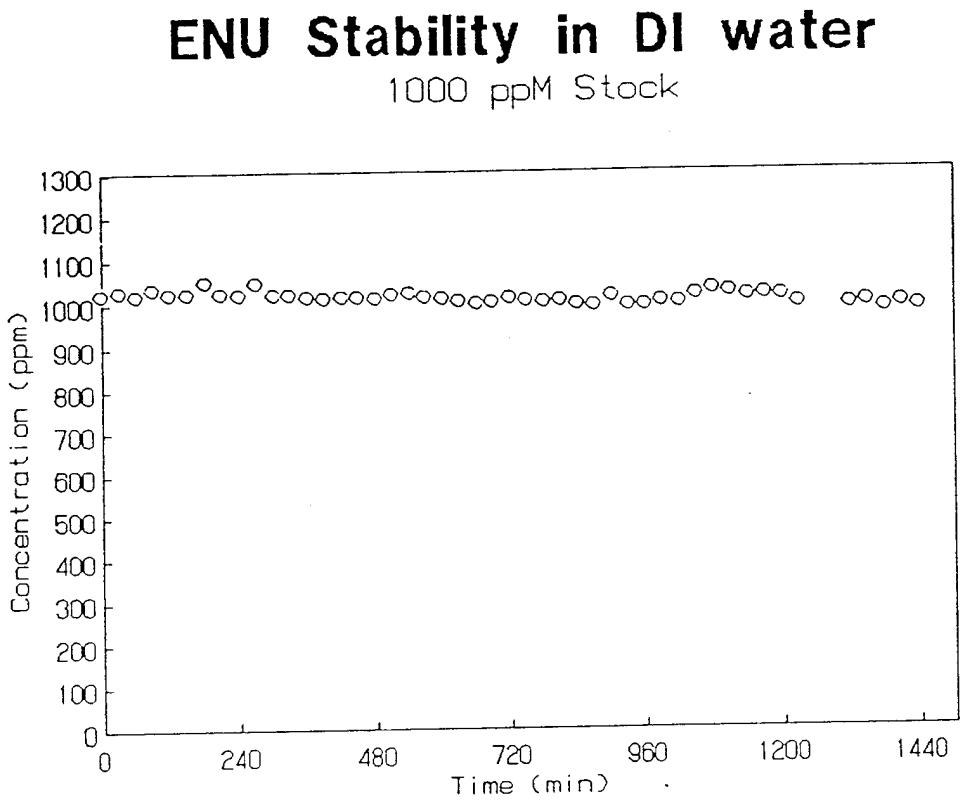


Figure 8. A plot of the stability of a 1000 mg/L N-Nitroso-N-ethylurea in deionized water over a 24 hour period.

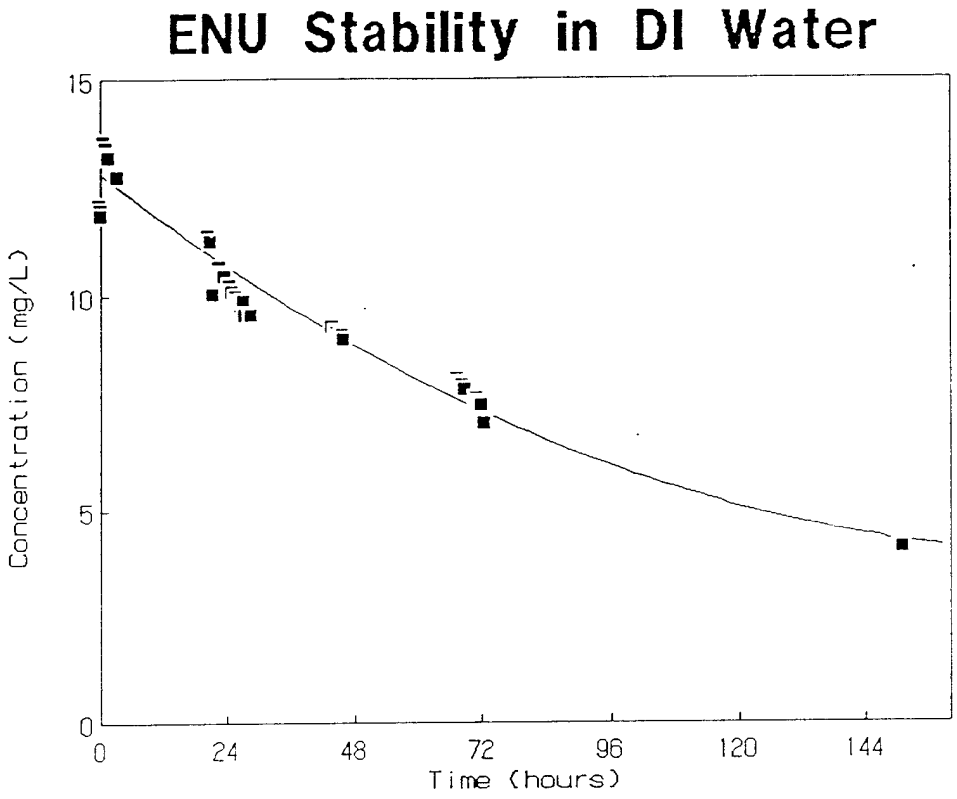


Figure 9. A plot of the stability of a 12.5 mg/L N-Nitroso-N-ethylurea in deionized water over a 7 day period.

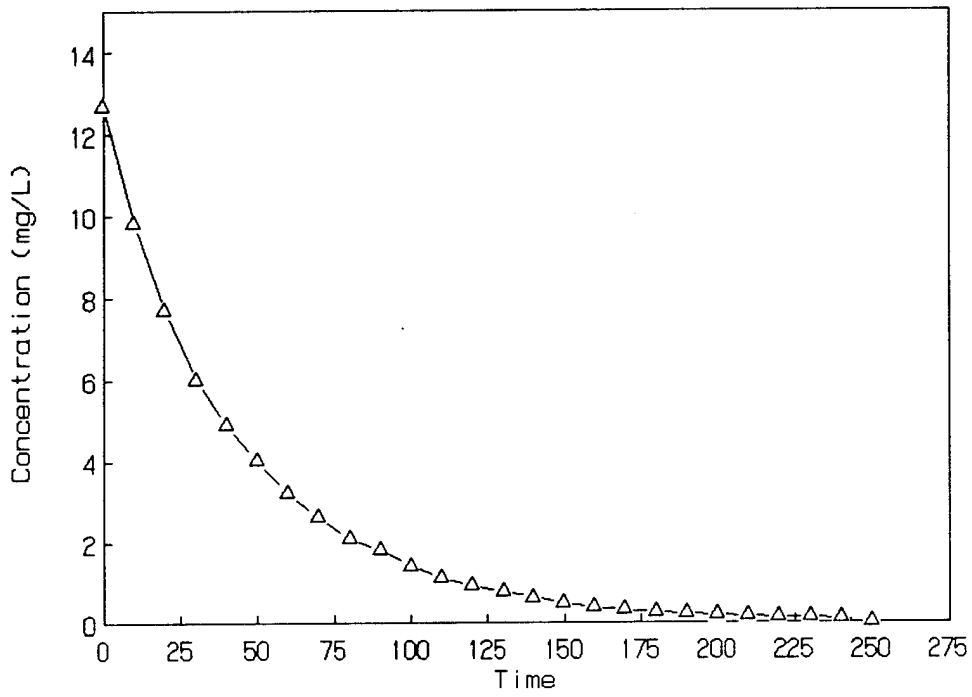


Figure 10. A plot of the stability of a well water sample spiked spiked with 26 mg/L N-Nitroso-N-ethylurea.

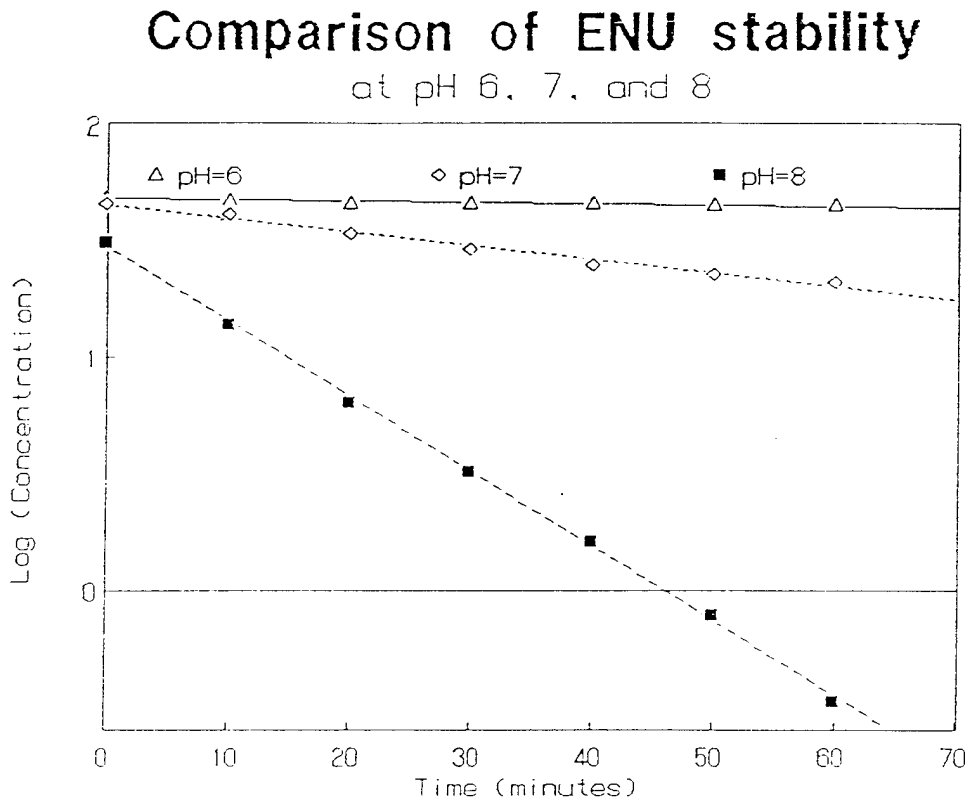


Figure 11. Plot of the of ENU stability at various pH values.

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